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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Donna T. Ward, William Gaarde, Brett P. Monia and Jacqueline Wyatt

For: **Antisense Modulation of MEKK4 Expression**

BOX SEQUENCE
Assistant Commissioner for Patents
Washington DC 20231

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find the following:

- The specification of the above-referenced patent application;
- An **executed** Declaration or Oath and Power of Attorney;
- An Assignment of the invention to **Isis Pharmaceuticals Inc.** with recordation cover sheet (PTO Form PTO-1595) and $40.00 cover fee;
- An **executed** Verified Statement Claiming Small Entity Status under 37 CFR 1.9 and 1.27;
- Statement to Support Filing and Submission of DNA/Amino Acid Sequences in Accordance with 37 CFR § § 1.821 through 1.825;
- Sequence listing in computer readable form in accordance with 37 C.F.R. § 1.821(e); and
- An Information Disclosure Statement with references.

The filing fee has been calculated as shown below:

For:	No. Filed	No. Extra	Rate	Fee
BASE FEE				$345.00
Total Claims	20 - 20 =	0	X $9=	$0
Indep. Claims	2 - 3 =	0	x$39=	$0
			TOTAL	$ 345.00

The Commissioner is hereby authorized to charge the following fees to Deposit Account No. 500252:

- the amount of $385.00 for the above listed fees;
- payment of the following fees associated with this communication or credit any overpayment;
- any additional filing fees required under 37 CFR 1.16 including fees for presentation of extra claims; and
- any additional patent application processing fees under 37 CFR 1.17 and under 37 CFR 1.20 (d).

Triplicate copies of this transmittal are enclosed.

Date: Sept 29, 2000

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Applicant or Patentee: Donna T. Ward, William Gaarde, Brett P. Monia and Jacqueline Wyatt

Serial or Patent No.: not yet assigned **Date Filed or Issued:** herewith

For: Antisense Modulation of MEKK4 Expression

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN)

I hereby declare that I am an official empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Isis Pharmaceuticals Inc.
ADDRESS OF CONCERN: 2292 Faraday Ave
Carlsbad, California 92008

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that: (1) the number of employees of the concern, including those of its affiliates, does not exceed 500 persons; and (2) the concern has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who could not be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section.

For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **Antisense Modulation of MEKK4 Expression** by inventor(s) Donna T. Ward, William Gaarde, Brett P. Monia and Jacqueline Wyatt described in the specification filed herewith.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

***NOTE: Separate verified statements are required for each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**

FULL NAME: **( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN**
ADDRESS: **( ) NONPROFIT ORGANIZATION**

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING C. Frank Bennett
TITLE OF PERSON SIGNING Vice President, Biology
ADDRESS OF PERSON SIGNING 2292 Faraday Avenue
Carlsbad, CA 92008

C. Frank Bennett
SIGNATURE

9-31-00
DATE

ANTISENSE MODULATION OF MEKK4 EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of MEKK4. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding MEKK4. Such compounds have been shown to modulate the expression of MEKK4.

BACKGROUND OF THE INVENTION

One of the principal mechanisms by which cellular regulation is effected is through the transduction of extracellular signals across the membrane that in turn modulate biochemical pathways within the cell. Protein phosphorylation represents one course by which intracellular signals are propagated from molecule to molecule resulting finally in a cellular response. These signal transduction cascades are highly regulated and often overlapping as evidenced by the existence of many protein kinases as well as protein phosphatases. It is currently believed that a number of disease states and/or disorders are a result of either aberrant expression or functional mutations in the molecular components of kinase cascades. Consequently, considerable attention has been devoted to the characterization of these proteins.

Nearly all cell surface receptors use one or more of the mitogen-activated protein kinase (MAP kinase) cascades during

signal transduction. Four distinct subgroups of the MAP kinases have been identified and each of these consists of a specific module of downstream kinases. One subgroup of the MAP kinases is the Jun N-terminal kinase/Stress activated protein kinase (JNK/SAPK) cascade. This pathway was originally identified as an oncogene- and ultraviolet light-stimulated kinase pathway but is now known to be activated by growth factors, cytokines, osmotic shock, wound stress and inflammatory factors (Moriguchi et al., *Adv. Pharmacol.*, **1996**, *36*, 121-137; Widmann et al., *Physiol. Rev.*, **1999**, *79*, 143-180).

MEKK4 (also known as mitogen-activated protein kinase kinase 4, MAP3K4, MAP Three Kinase 1, MAP/ERK kinase kinase 4, MAPKKK4 and MTK1) functions to mediate cellular responses to mitogenic stimuli within the JNK/SAPK signaling pathway (Gerwins et al., *J. Biol. Chem.*, **1997**, *272*, 8288-8295; Takekawa et al., *Embo J.*, **1997**, *16*, 4973-4982). First isolated in the mouse, MEKK4 is localized to perinuclear vesicular compartments and specifically activates the JNK pathway. In murine cell lines, MEKK4 has been shown to bind Cdc42 and Rac, two GTP binding proteins known to regulate pathways leading to the activation of the JNK pathway (Gerwins et al., *J. Biol. Chem.*, **1997**, *272*, 8288-8295).

In human cell lines, overexpression of MEKK4 activates the p38 MAP kinase pathway as well as the JNK pathway (Takekawa et al., *Embo J.*, **1997**, *16*, 4973-4982). The p38 pathway involves such cellular events as cytokine production and apoptosis, or programmed cell death, a tightly regulated process whose deregulation can result in a tumorigenic phenotype (Widmann et al., *Physiol. Rev.*, **1999**, *79*, 143-180). Furthermore, overexpression of a dominant negative form of MEKK4 in human cells results in the inhibition of activation of the p38 pathway by environmental stresses such as osmotic shock and ultraviolet irradiation (Takekawa et al., *Embo J.*, **1997**, *16*, 4973-4982). The pharmacological modulation of MEKK4 activity and/or expression may therefore be an appropriate point of therapeutic intervention in pathological conditions

resulting from environmental stress or injury such as inflammation and cancer.

In both mouse and human cells there exists at least two variants of the MEKK4 gene and each contains both a catalytic and regulatory domain. Northern blot analysis detected an approximately 6-kb MEKK4 transcript in various human tissues, with highest levels in heart, placenta, skeletal muscle, and pancreas. RT-PCR identified a shorter form of MEKK4 mRNA that lacks 49 codons and is probably generated by alternative splicing (Gerwins et al., *J. Biol. Chem.*, **1997**, *272*, 8288-8295; Takekawa et al., *Embo J.*, **1997**, *16*, 4973-4982).

Disclosed in US Patent 5,854,043 is the isolated MEKK4 protein as well as the delineation of the catalytic and regulatory domains of MEKK4 (Johnson, **1998**).

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of MEKK4 and to date, investigative strategies aimed at modulating MEKK4 function have involved the use of antibodies and compounds that block upstream entities that interfere in signal transduction pathways.

Disclosed in US Patent 5,910,417 are methods to treat allergic inflammation in humans, comprising administering an effective amount of a regulatory compound that interacts with a MEKK/JNKK signal transduction molecule from the group consisting of MEKK1, MEKK2, MEKK3, MEKK4, JNKK1, JNKK2 and JNK1. Further disclosed are methods to screen for the modulation of cytokine production after the treatment of hematopoietic cells with said regulatory compounds (Gelfand and Johnson, **1999**).

Disclosed in PCT application WO 98/54203 are methods to increase cancer cell sensitivity to cancer therapy by contacting said cells with a SAPK pathway inhibitor, specifically an inhibitor of MEKK1. These inhibitors being ribozymes, antisense nucleic acid molecules targeting a SAPK kinase kinase, or dominant negative mutants of an SAPK kinase kinase (Mercola, **1998**). However, within this PCT publication, the composition of these inhibitors is not

disclosed.

Disclosed in US Patent 5,981,265 are methods for regulating MEKK protein activity by transfecting or transforming a cell with a nucleic acid molecule capable of hybridizing with a nucleic acid molecule consisting of any of the known MEKK proteins, MEKK1, MEKK2, MEKK3, MEKK4, MEKK5 or MEKK6 (Johnson, **1999**).

However, these strategies are untested as therapeutic protocols. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting MEKK4 function.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of MEKK4 expression.

The present invention provides compositions and methods for modulating MEKK4 expression, including modulation of the alternatively spliced form of MEKK4.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding MEKK4, and which modulate the expression of MEKK4. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of MEKK4 in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of MEKK4 by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding MEKK4, ultimately modulating the amount of MEKK4 produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding MEKK4. As used herein, the terms "target nucleic acid" and "nucleic acid encoding MEKK4" encompass DNA encoding MEKK4, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of MEKK4. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is

associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding MEKK4. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding MEKK4, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and

"translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are

particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is

specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic

modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to

either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside

residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and

5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or

O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_2)_2$, also described in examples hereinbelow.

A further prefered modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 3' or 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-$CH=CH_2$), 2'-O-allyl (2'-O-CH_2-$CH=CH_2$) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is

2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified

nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273;

5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-

1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;

4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA

target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844;

5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline,

diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic

acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of MEKK4 is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the

antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding MEKK4, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding MEKK4 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of MEKK4 in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be

necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers

surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Prefered bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate,. Prefered fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also prefered are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly prefered combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-

hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The

compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets

into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of

emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., **1988**, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1,

p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*,

Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, *in: Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate

microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385; Ho *et al.*, *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating

non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations

are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al., Biochem. Biophys. Res. Commun.,* ***1987,*** *147*, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than

complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, **1992**, *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.* as a solution or as an emulsion) were ineffective (Weiner *et al.*, *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-

10-stearyl ether) and Novasome™ II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P.Pharma. Sci.*, **1994**, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters,* ***1987,*** *223*, 42; Wu *et al.*, *Cancer Research,* **1993,** *53*, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, **1987,** *507*, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, **1988,** *85*, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes

comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, **1980,** *53,* 2778) described liposomes comprising a nonionic detergent, $2C_{12}15G$, that contains a PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984**, *167,* 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*, **1990**, *268,* 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta,* **1990,** *1029,* 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.* they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical*

Dosage Forms, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., **1992**, *44*, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier*

Systems, **1991**, page 92; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, **1992**, *263*, 25; Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, *79*, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Buur *et al.*, *J. Control Rel.*, **1990**, *14*, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone

derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate,

polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir,

may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance

doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard

methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of

product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and

2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated

with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was

chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried

(40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-*O*-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was strirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and

stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and

concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH_2-O-CH_2-N$(CH_2)_2$, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy) ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl

solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH_2Cl_2:Et_3N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated

synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-

methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate

internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid

expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 5 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg,

MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

HepG2 cells:

The human hepatoblastoma cell line HepG2 was obtained from the American Type Culure Collection (Manassas, VA). HepG2 cells were routinely cultured in Eagle's MEM supplemented with 10% fetal calf serum, non-essential amino acids, and 1 mM sodium pyruvate (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates,

wells were washed once with 200 µL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

Example 10

Analysis of oligonucleotide inhibition of MEKK4 expression

Antisense modulation of MEKK4 expression can be assayed in a variety of ways known in the art. For example, MEKK4 mRNA levels can be quantitated by, e.g., Northern blot

analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.*M. et al., Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of MEKK4 can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to MEKK4 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in*

Molecular Biology, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., **1991**.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, **1996**, *42*, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 µL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 µL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 100 µL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 µL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13

Real-time Quantitative PCR Analysis of MEKK4 mRNA Levels

Quantitation of MEKK4 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after

antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM $MgCl_2$, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA

using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, *Analytical Biochemistry*, **1998**, *265*, 368-374.

In this assay, 175 µL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human MEKK4 were designed to hybridize to a human MEKK4 sequence, using published sequence information (GenBank accession number D86968, incorporated herein as SEQ ID NO:3). For human MEKK4 the PCR primers were:
forward primer: ACTCCTGGAACAAAGATTGTAGGTTACT (SEQ ID NO: 4)
reverse primer: CTCTAGCAGCTCCATTATCCGTTT (SEQ ID NO: 5) and the PCR probe was: FAM-TCTCCAACGCCAGAGGGTCTCATTTG-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were:
forward primer: CAACGGATTTGGTCGTATTGG (SEQ ID NO: 7)
reverse primer: GGCAACAATATCCACTTTACCAGAGT (SEQ ID NO: 8) and the PCR probe was: 5' JOE-CGCCTGGTCACCAGGGCTGCT- TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of MEKK4 mRNA levels

Eighteen hours after antisense treatment, cell

monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then robed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human MEKK4, a human MEKK4 specific probe was prepared by PCR using the forward primer ACTCCTGGAACAAAGATTGTAGGTTACT (SEQ ID NO: 4) and the reverse primer CTCTAGCAGCTCCATTATCCGTTT (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

Antisense inhibition of human MEKK4 expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of

the human MEKK4 RNA, using published sequences (GenBank accession number D86968, incorporated herein as SEQ ID NO: 3, GenBank accession number AF002715, incorporated herein as SEQ ID NO: 10, and GenBank accession number AA669565, the complement of which is incorporated herein as SEQ ID NO: 11). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human MEKK4 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human MEKK4 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
123085	Coding	3	3314	gctggaacccctgaatccct	30	12
123086	5'UTR	10	37	cgactccgcctccgcctcta	20	13
123087	5'UTR	10	47	gggagtgactcgactccgcc	2	14
123088	Start Codon	10	134	gcttctctcatccgtgcacg	39	15
123089	Coding	10	267	caagcagcactcgggttctg	30	16
123090	Coding	10	327	tagatcagattcaggactct	62	17
123091	Coding	10	377	ggagaggtaccataaagatt	0	18
123092	Coding	10	401	ttcatctgtcgaggtgtgct	0	19
123093	Coding	10	436	ccacattattcctctgatgt	0	20
123094	Coding	10	444	tggcctccccacattattcc	5	21
123095	Coding	10	467	tctttcaaattagaccgact	47	22
123096	Coding	10	677	ttgagatccacatctggaat	0	23

123097	Coding	10	687	gtaaggcttattgagatcca	36	24
123098	Coding	10	833	gaggtaagctttagcaaaag	19	25
123099	Coding	10	923	tctaaccagatcagttcgtt	50	26
123100	Coding	10	1261	tttcatagtccttctgaaga	34	27
123101	Coding	10	1274	tttgcagcatatttttcata	20	28
123102	Coding	10	1398	tggccagccaatgtctgata	54	29
123103	Coding	10	1408	tttcaaacactggccagcca	43	30
123104	Coding	10	1443	cggctcattacctttggatg	20	31
123105	Coding	10	1478	tttaattctccttctgtgtc	48	32
123106	Coding	10	1875	ctgcacataatctgaacccc	25	33
123107	Coding	10	1889	ggtgtccttgacaactgcac	37	34
123108	Coding	10	2147	tcctgcagcatgaactggta	41	35
123109	Coding	10	2237	atgtaatcaaaatacaccat	36	36
123110	Coding	10	2247	ccagcttctcatgtaatcaa	2	37
123111	Coding	10	2257	gcatttggatccagcttctc	17	38
123112	Coding	10	2293	tttttaaactatgcgatgct	38	39
123113	Coding	10	2327	tctttggtgaaattccattc	56	40
123114	Coding	10	2511	ctctataacagacctgatga	0	41
123115	Coding	10	2606	atttccaggtccttttctcaa	47	42
123116	Coding	10	2676	gacatactgttttgatttca	23	43
123117	Coding	10	2768	gcattgagtaactgcaaaat	6	44
123118	Coding	10	2924	acagtctccacctgaggcac	6	45
123119	Coding	10	2932	gggtgtcaacagtctccacc	76	46
123120	Coding	10	2945	tgcatgctttctcagggtgtc	29	47
123121	Coding	10	3026	ccctcaatggactgctggaa	48	48
123122	Coding	10	3133	cattgcttatcctgttgcat	39	49
123123	Coding	10	3272	acttctttatgatactcaaa	8	50
123124	Coding	10	3282	caaacgaacaacttctttat	45	51
123125	Coding	10	3292	ccccagacatcaaacgaaca	0	52
123126	Coding	10	3320	tatttgtctcctatcttctg	66	53
123127	Coding	10	3338	ttccgggcaaagcttatata	18	54
123128	Coding	10	3346	tcatccacttccgggcaaag	0	55
123129	Coding	10	3395	cacctgggtcttgtacctct	38	56
123130	Coding	10	3446	gaaataaaggcaggttcaat	11	57
123131	Coding	10	3456	tggtaaagctgaaataaagg	0	58
123132	Coding	10	3466	agtcatcttctggtaaagct	0	59
123133	Coding	10	3476	aaactcaagaagtcatcttc	39	60
123134	Coding	10	3650	gtgctgaatccctctggagt	13	61
123135	Coding	10	3678	gctccgcgcgtcggaaggca	28	62
123136	Coding	10	3710	gcagcagcagcagcagcagc	49	63
123137	Coding	10	3804	gctggaacccctggtatcat	22	64
123138	Coding	10	3840	agcagctatggaagccaatc	41	65
123139	Coding	10	4016	ttctggatagcttctatggg	28	66
123140	Coding	10	4026	tcggactgacttctggatag	69	67
123141	Coding	10	4073	atgatattctttctcctcat	27	68
123142	Coding	10	4166	attttgtttcctctttgcca	52	69
123143	Coding	10	4235	ttcatggccatcagctcccc	18	70
123144	Coding	10	4352	tggagctccacaccaaaata	11	71
123145	Coding	10	4382	tactccatgaagatgtacat	3	72
123146	Coding	10	4490	tgctcatggaggacgttgat	3	73
123147	Coding	10	4532	aggaagatattggcaccttt	37	74
123148	Coding	10	4548	taatccagatgaggtaagga	11	75
123149	Coding	10	4560	tcccagtttgattaatccag	23	76
123150	Coding	10	4589	tttttgagctttactgaaca	26	77
123151	Coding	10	4742	ccagtcaccatctctatgac	0	78
123152	Coding	10	4913	tggtcgaggagctggctggc	48	79
123153	Coding	10	4937	tctgtgcaaaccttgacaaa	5	80

123154	Stop Codon	10	4955	actaggcttcattcttcatc	0	81
123155	3'UTR	10	5002	atattacatacagtagtgat	19	82
123156	3'UTR	10	5012	ctttatgtaaatattacata	13	83
123157	3'UTR	10	5154	ctttaacctcgtggccacca	18	84
123158	3'UTR	10	5172	gcacttaacatgcagcttct	0	85
123159	3'UTR	10	5183	cagtagtaatggcacttaac	20	86
123160	3'UTR	10	5384	aacctgcagcttgccaacaa	57	87
123161	3'UTR	10	5407	agtaatcagccttttgcatt	7	88
123162	Exon	11	67	agaacctttttcttaaattt	36	89

As shown in Table 1, SEQ ID NOs 12, 13, 15, 16, 17, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 39, 40, 42, 43, 46, 47, 48, 49, 51, 53, 54, 56, 60, 62, 63, 64, 65, 66, 67, 68, 69, 70, 74, 76, 77, 79, 82, 84, 86, 87 and 89 demonstrated at least 15% inhibition of human MEKK4 expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

Example 16

Western blot analysis of MEKK4 protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to MEKK4 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

What is claimed is:

1. A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding MEKK4, wherein said compound specifically hybridizes with and inhibits the expression of MEKK4.

2. The compound of claim 1 which is an antisense oligonucleotide.

3. The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 12, 13, 15, 16, 17, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 39, 40, 42, 43, 46, 47, 48, 49, 51, 53, 54, 56, 60, 62, 63, 64, 65, 66, 67, 68, 69, 70, 74, 76, 77, 79, 82, 84, 86, 87 or 89.

4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

5. The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.

6. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

7. The compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

8. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

9. The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.

10. The compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

11. A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding MEKK4.

12. A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a

colloidal dispersion system.

14. The composition of claim 12 wherein the compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of MEKK4 in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of MEKK4 is inhibited.

16. A method of treating an animal having a disease or condition associated with MEKK4 comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of MEKK4 is inhibited.

17. The method of claim 16 wherein the disease or condition is an immunologic disorder.

18. The method of claim 16 wherein the disease or condition is an inflammatory disorder.

19. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

20. The method of claim 19 wherein the hyperproliferative disorder is cancer.

ABSTRACT

Antisense compounds, compositions and methods are provided for modulating the expression of MEKK4. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding MEKK4. Methods of using these compounds for modulation of MEKK4 expression and for treatment of diseases associated with expression of MEKK4 are provided.

DOCKET NO.: RTS-0169

"Express Mail" Label No.: EL263653993US
Date of Deposit: 9-29-00

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **Antisense Modulation of MEKK4 Expression** the specification of which:

(XX) is attached hereto.

( ) was filed on_____as Application Serial No._____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed			
			Yes		No	
			Yes		No	
			Yes		No	

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented)

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Provisional Application No.	Filing Date

DOCKET NO.: RTS-0169

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Herb Boswell, Registration No. 27,311; Laurel Spear Bernstein, Registration No. 37,280 and April C. Logan, Registration No. 33,950, of Isis Pharmaceuticals, Inc.; and Jane Massey Licata, Registration No. 32,257, and Kathleen A. Tyrrell, Registration No. 38,350 of the firm of Law Offices of Jane Massey Licata, 66 East Main Street, Marlton NJ 08053.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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<110> Donna T. Ward
William Gaarde
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<120> ANTISENSE MODULATION OF MEKK4 EXPRESSION

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tct ccc ccc agc aca cct cga cag atg aaa cgc atg tca acc aaa cat     96
Ser Pro Pro Ser Thr Pro Arg Gln Met Lys Arg Met Ser Thr Lys His
            20                  25                  30

cag agg aat aat gtg ggg agg cca gcc agt cgg tct aat ttg aaa gaa    144
Gln Arg Asn Asn Val Gly Arg Pro Ala Ser Arg Ser Asn Leu Lys Glu
        35                  40                  45

aaa atg aat gca cca aat cag cct cca cat aaa gac act gga aaa aca    192
Lys Met Asn Ala Pro Asn Gln Pro Pro His Lys Asp Thr Gly Lys Thr
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gtg gag aat gtg gaa gaa tac agc tat aag cag gag aaa aag atc cga    240
Val Glu Asn Val Glu Glu Tyr Ser Tyr Lys Gln Glu Lys Lys Ile Arg
65                  70                  75                  80

gca gct ctt aga aca aca gag cgt gat cat aaa aaa aat gta cag tgc    288
Ala Ala Leu Arg Thr Thr Glu Arg Asp His Lys Lys Asn Val Gln Cys
                85                  90                  95

tca ttc atg tta gac tca gtg ggt gga tct ttg cca aaa aaa tca att    336
Ser Phe Met Leu Asp Ser Val Gly Gly Ser Leu Pro Lys Lys Ser Ile
            100                 105                 110

cca gat gtg gat ctc aat aag cct tac ctc agc ctt ggc tgt agc aat    384
Pro Asp Val Asp Leu Asn Lys Pro Tyr Leu Ser Leu Gly Cys Ser Asn
        115                 120                 125

gct aag ctt cca gta tct gtg ccc atg cct ata gcc aga cct gca cgc    432
Ala Lys Leu Pro Val Ser Val Pro Met Pro Ile Ala Arg Pro Ala Arg
    130                 135                 140

cag act tct agg act gac tgt cca gca gat cgt tta aag ttt ttt gaa    480
Gln Thr Ser Arg Thr Asp Cys Pro Ala Asp Arg Leu Lys Phe Phe Glu
145                 150                 155                 160
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act tta cga ctt ttg cta aag ctt acc tca gtc tca aag aaa aaa gac      528
Thr Leu Arg Leu Leu Leu Lys Leu Thr Ser Val Ser Lys Lys Lys Asp
                165                 170                 175

agg gag caa aga gga caa gaa aat acg tct ggt ttc tgg ctt aac cga      576
Arg Glu Gln Arg Gly Gln Glu Asn Thr Ser Gly Phe Trp Leu Asn Arg
            180                 185                 190

tct aac gaa ctg atc tgg tta gag cta caa gcc tgg cat gca gga cgg      624
Ser Asn Glu Leu Ile Trp Leu Glu Leu Gln Ala Trp His Ala Gly Arg
        195                 200                 205

aca att aac gac cag gac ttc ttt tta tat aca gcc cgt caa gcc atc      672
Thr Ile Asn Asp Gln Asp Phe Phe Leu Tyr Thr Ala Arg Gln Ala Ile
    210                 215                 220

cca gat att att aat gaa atc ctt act ttc aaa gtc gac tat ggg agc      720
Pro Asp Ile Ile Asn Glu Ile Leu Thr Phe Lys Val Asp Tyr Gly Ser
225                 230                 235                 240

ttc gcc ttt gtt aga gat aga gct ggt ttt aat ggt act tca gta gaa      768
Phe Ala Phe Val Arg Asp Arg Ala Gly Phe Asn Gly Thr Ser Val Glu
                245                 250                 255

ggg cag tgc aaa gcc act cct gga aca aag att gta ggt tac tca aca      816
Gly Gln Cys Lys Ala Thr Pro Gly Thr Lys Ile Val Gly Tyr Ser Thr
            260                 265                 270

cat cat gag cat ctc caa cgc cag agg gtc tca ttt gag cag gta aaa      864
His His Glu His Leu Gln Arg Gln Arg Val Ser Phe Glu Gln Val Lys
        275                 280                 285

cgg ata atg gag ctg cta gag tac ata gaa gca ctt tat cca tca ttg      912
Arg Ile Met Glu Leu Leu Glu Tyr Ile Glu Ala Leu Tyr Pro Ser Leu
    290                 295                 300

cag gct ctt cag aag gac tat gaa aaa tat gct gca aaa gac ttc cag      960
Gln Ala Leu Gln Lys Asp Tyr Glu Lys Tyr Ala Ala Lys Asp Phe Gln
305                 310                 315                 320

gac agg gtg cag gca ctc tgt ttg tgg tta aac atc aca aaa gac tta     1008
Asp Arg Val Gln Ala Leu Cys Leu Trp Leu Asn Ile Thr Lys Asp Leu
                325                 330                 335
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aat cag aaa tta agg att atg ggc act gtt ttg ggc atc aag aat tta    1056
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tca gac att ggc tgg cca gtg ttt gaa atc cct tcc cct cga cca tcc    1104
Ser Asp Ile Gly Trp Pro Val Phe Glu Ile Pro Ser Pro Arg Pro Ser
        355                 360                 365

aaa ggt aat gag ccg gag tat gag ggt gat gac aca gaa gga gaa tta    1152
Lys Gly Asn Glu Pro Glu Tyr Glu Gly Asp Asp Thr Glu Gly Glu Leu
    370                 375                 380

aag gag ttg gaa agt agt acg gat gag agt gaa gaa gaa caa atc tct    1200
Lys Glu Leu Glu Ser Ser Thr Asp Glu Ser Glu Glu Glu Gln Ile Ser
385                 390                 395                 400

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Asp Pro Arg Val Pro Glu Ile Arg Gln Pro Ile Asp Asn Ser Phe Asp
                405                 410                 415

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Ile Gln Ser Arg Asp Cys Ile Ser Lys Lys Leu Glu Arg Leu Glu Ser
            420                 425                 430

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Glu Asp Asp Ser Leu Gly Trp Gly Ala Pro Asp Trp Ser Thr Glu Ala
        435                 440                 445

ggc ttt agt aga cat tgt ctg act tct att tat aga cca ttt gta gac    1392
Gly Phe Ser Arg His Cys Leu Thr Ser Ile Tyr Arg Pro Phe Val Asp
    450                 455                 460

aaa gca ctg aag cag atg ggg tta aga aag tta att tta aga ctt cac    1440
Lys Ala Leu Lys Gln Met Gly Leu Arg Lys Leu Ile Leu Arg Leu His
465                 470                 475                 480

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Lys Leu Met Asp Gly Ser Leu Gln Arg Ala Arg Ile Ala Leu Val Lys
                485                 490                 495

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Asn Asp Arg Pro Val Glu Phe Ser Glu Phe Pro Asp Pro Met Trp Gly
            500                 505                 510
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        515                 520                 525

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Cys Ser Ala Val Ser Trp Glu Glu Leu Lys Ala Met Asp Leu Pro Ser
    530                 535                 540

ttc gaa cct gcc ttc cta gtt ctc tgc cga gtc ctt ctg aat gtc ata    1680
Phe Glu Pro Ala Phe Leu Val Leu Cys Arg Val Leu Leu Asn Val Ile
545                 550                 555                 560

cat gag tgt ctg aag tta aga ttg gag cag aga cct gct gga gaa cca    1728
His Glu Cys Leu Lys Leu Arg Leu Glu Gln Arg Pro Ala Gly Glu Pro
                565                 570                 575

tct ctc ttg agt att aag cag ctg gtg aga gag tgt aag gag gtc ctg    1776
Ser Leu Leu Ser Ile Lys Gln Leu Val Arg Glu Cys Lys Glu Val Leu
            580                 585                 590

aag ggc ggc ctg ctg atg aag cag tac tac cag ttc atg ctg cag gag    1824
Lys Gly Gly Leu Leu Met Lys Gln Tyr Tyr Gln Phe Met Leu Gln Glu
        595                 600                 605

gtt ctg gag gac ttg gag aag ccc gac tgc aac att gac gct ttt gaa    1872
Val Leu Glu Asp Leu Glu Lys Pro Asp Cys Asn Ile Asp Ala Phe Glu
    610                 615                 620

gag gat cta cat aaa atg ctt atg gtg tat ttt gat tac atg aga agc    1920
Glu Asp Leu His Lys Met Leu Met Val Tyr Phe Asp Tyr Met Arg Ser
625                 630                 635                 640

tgg atc caa atg cta cag caa tta cct caa gca tcg cat agt tta aaa    1968
Trp Ile Gln Met Leu Gln Gln Leu Pro Gln Ala Ser His Ser Leu Lys
                645                 650                 655

aat ctg tta gaa gaa gaa tgg aat ttc acc aaa gaa ata act cat tac    2016
Asn Leu Leu Glu Glu Glu Trp Asn Phe Thr Lys Glu Ile Thr His Tyr
            660                 665                 670

ata cgg gga gga gaa gca cag gcc ggg aag ctt ttc tgt gac att gca    2064
Ile Arg Gly Gly Glu Ala Gln Ala Gly Lys Leu Phe Cys Asp Ile Ala
        675                 680                 685
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gga atg ctg ctg aaa tct aca gga agt ttt tta gaa ttt ggc tta cag    2112
Gly Met Leu Leu Lys Ser Thr Gly Ser Phe Leu Glu Phe Gly Leu Gln
    690                 695                 700

gag agc tgt gct gaa ttt tgg act agt gcg gat gac agc agt gct tcc    2160
Glu Ser Cys Ala Glu Phe Trp Thr Ser Ala Asp Asp Ser Ser Ala Ser
705                 710                 715                 720

gac gaa atc agg agg tct gtt ata gag atc agt cga gcc ctg aag gag    2208
Asp Glu Ile Arg Arg Ser Val Ile Glu Ile Ser Arg Ala Leu Lys Glu
                725                 730                 735

ctc ttc cat gaa gcc aga gaa agg gct tcc aaa gca ctt gga ttt gct    2256
Leu Phe His Glu Ala Arg Glu Arg Ala Ser Lys Ala Leu Gly Phe Ala
            740                 745                 750

aaa atg ttg aga aag gac ctg gaa ata gca gca gaa ttc agg ctt tca    2304
Lys Met Leu Arg Lys Asp Leu Glu Ile Ala Ala Glu Phe Arg Leu Ser
        755                 760                 765

gcc cca gtt aga gac ctc ctg gat gtt ctg aaa tca aaa cag tat gtc    2352
Ala Pro Val Arg Asp Leu Leu Asp Val Leu Lys Ser Lys Gln Tyr Val
    770                 775                 780

aag gtg caa att cct ggg tta gaa aac ttg caa atg ttt gtt cca gac    2400
Lys Val Gln Ile Pro Gly Leu Glu Asn Leu Gln Met Phe Val Pro Asp
785                 790                 795                 800

act ctt gct gag gag aag agt att att ttg cag tta ctc aat gca gct    2448
Thr Leu Ala Glu Glu Lys Ser Ile Ile Leu Gln Leu Leu Asn Ala Ala
                805                 810                 815

gca gga aag gac tgt tca aaa gat tca gat gac gta ctc atc gat gcc    2496
Ala Gly Lys Asp Cys Ser Lys Asp Ser Asp Asp Val Leu Ile Asp Ala
            820                 825                 830

tat ctg ctt ctg acc aag cac ggt gat cga gcc cgt gat tca gag gac    2544
Tyr Leu Leu Leu Thr Lys His Gly Asp Arg Ala Arg Asp Ser Glu Asp
        835                 840                 845

agc tgg ggc acc tgg gag gca cag cct gtc aaa gtc gtg cct cag gtg    2592
Ser Trp Gly Thr Trp Glu Ala Gln Pro Val Lys Val Val Pro Gln Val
    850                 855                 860
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gag act gtt gac acc ctg aga agc atg cag gtg gat aat ctt tta cta    2640
Glu Thr Val Asp Thr Leu Arg Ser Met Gln Val Asp Asn Leu Leu Leu
865                 870                 875                 880

gtt gtc atg cag tct gcg cat ctc aca att cag aga aaa gct ttc cag    2688
Val Val Met Gln Ser Ala His Leu Thr Ile Gln Arg Lys Ala Phe Gln
                885                 890                 895

cag tcc att gag gga ctt atg act ctg tgc cag gag cag aca tcc agt    2736
Gln Ser Ile Glu Gly Leu Met Thr Leu Cys Gln Glu Gln Thr Ser Ser
            900                 905                 910

cag ccg gtc atc gcc aaa gct ttg cag cag ctg aag aat gat gca ttg    2784
Gln Pro Val Ile Ala Lys Ala Leu Gln Gln Leu Lys Asn Asp Ala Leu
        915                 920                 925

gag cta tgc aac agg ata agc aat gcc att gac cgc gtg gac cac atg    2832
Glu Leu Cys Asn Arg Ile Ser Asn Ala Ile Asp Arg Val Asp His Met
    930                 935                 940

ttc aca tca gaa ttt gat gct gag gtt gat gaa tct gaa tct gtc acc    2880
Phe Thr Ser Glu Phe Asp Ala Glu Val Asp Glu Ser Glu Ser Val Thr
945                 950                 955                 960

ttg caa cag tac tac cga gaa gca atg att cag ggg tac aat ttt gga    2928
Leu Gln Gln Tyr Tyr Arg Glu Ala Met Ile Gln Gly Tyr Asn Phe Gly
                965                 970                 975

ttt gag tat cat aaa gaa gtt gtt cgt ttg atg tct ggg gag ttt aga    2976
Phe Glu Tyr His Lys Glu Val Val Arg Leu Met Ser Gly Glu Phe Arg
            980                 985                 990

cag aag ata gga gac aaa tat ata agc ttt gcc cgg aag tgg atg aat    3024
Gln Lys Ile Gly Asp Lys Tyr Ile Ser Phe Ala Arg Lys Trp Met Asn
        995                 1000                1005

tat gtc ctg act aaa tgt gag agt ggt aga ggt aca aga ccc agg tgg    3072
Tyr Val Leu Thr Lys Cys Glu Ser Gly Arg Gly Thr Arg Pro Arg Trp
    1010                1015                1020

gcg act caa gga ttt gat ttt cta caa gca att gaa cct gcc ttt att    3120
Ala Thr Gln Gly Phe Asp Phe Leu Gln Ala Ile Glu Pro Ala Phe Ile
1025                1030                1035                1040
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tca gct tta cca gaa gat gac ttc ttg agt tta caa gcc ttg atg aat    3168
Ser Ala Leu Pro Glu Asp Asp Phe Leu Ser Leu Gln Ala Leu Met Asn
                1045                1050                1055

gaa tgc att ggc cat gtc ata gga aaa cca cac agt cct gtt aca ggt    3216
Glu Cys Ile Gly His Val Ile Gly Lys Pro His Ser Pro Val Thr Gly
            1060                1065                1070

ttg tac ctt gcc att cat cgg aac agc ccc cgt cct atg aag gta cct    3264
Leu Tyr Leu Ala Ile His Arg Asn Ser Pro Arg Pro Met Lys Val Pro
        1075                1080                1085

cga tgc cat agt gac cct cct aac cca cac ctc att atc ccc act cca    3312
Arg Cys His Ser Asp Pro Pro Asn Pro His Leu Ile Ile Pro Thr Pro
    1090                1095                1100

gag gga ttc agg ggt tcc agc gtt cct gaa aat gat cga ttg gct tcc    3360
Glu Gly Phe Arg Gly Ser Ser Val Pro Glu Asn Asp Arg Leu Ala Ser
1105                1110                1115                1120

ata gct gct gaa ttg cag ttt agg tcc ctg agt cgt cac tca agc ccc    3408
Ile Ala Ala Glu Leu Gln Phe Arg Ser Leu Ser Arg His Ser Ser Pro
                1125                1130                1135

acg gag gag cga gat gaa cca gca tat cca aga gga gat tca agt ggg    3456
Thr Glu Glu Arg Asp Glu Pro Ala Tyr Pro Arg Gly Asp Ser Ser Gly
            1140                1145                1150

tcc aca aga aga agt tgg gaa ctt cgg aca cta atc agc cag agt aaa    3504
Ser Thr Arg Arg Ser Trp Glu Leu Arg Thr Leu Ile Ser Gln Ser Lys
        1155                1160                1165

gat act gct tct aaa cta gga ccc ata gaa gct atc cag aag tca gtc    3552
Asp Thr Ala Ser Lys Leu Gly Pro Ile Glu Ala Ile Gln Lys Ser Val
    1170                1175                1180

cga ttg ttt gaa gaa aag agg tac cga gaa atg agg aga aag aat atc    3600
Arg Leu Phe Glu Glu Lys Arg Tyr Arg Glu Met Arg Arg Lys Asn Ile
1185                1190                1195                1200

att ggt caa gtt tgt gat acg cct aag tcc tat gat aat gtt atg cac    3648
Ile Gly Gln Val Cys Asp Thr Pro Lys Ser Tyr Asp Asn Val Met His
                1205                1210                1215
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gtt ggc ttg agg aag gtg acc ttc aaa tgg caa aga gga aac aaa att    3696
Val Gly Leu Arg Lys Val Thr Phe Lys Trp Gln Arg Gly Asn Lys Ile
            1220                1225                1230

gga gaa ggc cag tat ggg aag gtg tac acc tgc atc agc gtc gac acc    3744
Gly Glu Gly Gln Tyr Gly Lys Val Tyr Thr Cys Ile Ser Val Asp Thr
        1235                1240                1245

ggg gag ctg atg gcc atg aaa gag att cga ttt caa cct aat gac cat    3792
Gly Glu Leu Met Ala Met Lys Glu Ile Arg Phe Gln Pro Asn Asp His
    1250                1255                1260

aag act atc aag gaa act gca gac gaa ttg aaa ata ttc gaa ggc atc    3840
Lys Thr Ile Lys Glu Thr Ala Asp Glu Leu Lys Ile Phe Glu Gly Ile
1265                1270                1275                1280

aaa cac ccc aat ctg gtt cgg tat ttt ggt gtg gag ctc cat aga gaa    3888
Lys His Pro Asn Leu Val Arg Tyr Phe Gly Val Glu Leu His Arg Glu
                1285                1290                1295

gaa atg tac atc ttc atg gag tac tgc gat gag ggg act tta gaa gag    3936
Glu Met Tyr Ile Phe Met Glu Tyr Cys Asp Glu Gly Thr Leu Glu Glu
            1300                1305                1310

gtg tca agg ctg gga ctt cag gaa cat gtg att agg ctg tat tca aag    3984
Val Ser Arg Leu Gly Leu Gln Glu His Val Ile Arg Leu Tyr Ser Lys
        1315                1320                1325

cag atc acc att gcg atc aac gtc ctc cat gag cat ggc ata gtc cac    4032
Gln Ile Thr Ile Ala Ile Asn Val Leu His Glu His Gly Ile Val His
    1330                1335                1340

cgt gac att aaa ggt gcc aat atc ttc ctt acc tca tct gga tta atc    4080
Arg Asp Ile Lys Gly Ala Asn Ile Phe Leu Thr Ser Ser Gly Leu Ile
1345                1350                1355                1360

aaa ctg gga gat ttt gga tgt tca gta aag ctc aaa aac aat gcc cag    4128
Lys Leu Gly Asp Phe Gly Cys Ser Val Lys Leu Lys Asn Asn Ala Gln
                1365                1370                1375

acc atg cct ggt gaa gtg aac agc acc ctg ggg aca gca gca tac atg    4176
Thr Met Pro Gly Glu Val Asn Ser Thr Leu Gly Thr Ala Ala Tyr Met
            1380                1385                1390
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gca cct gaa gtc atc act cgt gcc aaa gga gag ggc cat ggg cgt gcg    4224
Ala Pro Glu Val Ile Thr Arg Ala Lys Gly Glu Gly His Gly Arg Ala
        1395                1400                1405

gcc gac atc tgg agt ctg ggg tgt gtt gtc ata gag atg gtg act ggc    4272
Ala Asp Ile Trp Ser Leu Gly Cys Val Val Ile Glu Met Val Thr Gly
    1410                1415                1420

aag agg cct tgg cat gag tat gag cac aac ttt caa att atg tat aaa    4320
Lys Arg Pro Trp His Glu Tyr Glu His Asn Phe Gln Ile Met Tyr Lys
1425                1430                1435                1440

gtg ggg atg gga cat aag cca cca atc cct gaa aga tta agc cct gaa    4368
Val Gly Met Gly His Lys Pro Pro Ile Pro Glu Arg Leu Ser Pro Glu
                1445                1450                1455

gga aag gac ttc ctt tct cac tgc ctt gag agt gac cca aag atg aga    4416
Gly Lys Asp Phe Leu Ser His Cys Leu Glu Ser Asp Pro Lys Met Arg
            1460                1465                1470

tgg acc gcc agc cag ctc ctc gac cat tcg ttt gtc aag gtt tgc aca    4464
Trp Thr Ala Ser Gln Leu Leu Asp His Ser Phe Val Lys Val Cys Thr
        1475                1480                1485

gat gaa gaa tga agcctagtag aatatggact tggaaaattc tcttaatcac        4516
Asp Glu Glu XXX
    1490
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tactgtatgt aatatttaca taaagactgt gctgagaagc agtataagcc tttttaacct 4576

tccaagactg aagactgcac aggtgacaag cgtcacttct cctgctgctc ctgtttgtct 4636

gatgtggcaa aaggccctct ggagggctgg tggccacgag gttaaagaag ctgcatgtta 4696

agtgccatta ctactgtaca cggaccatcg cctctgtctc ctccgtgtct cgcgcgactg 4756

agaaccgtga catcagcgta gtgttttgac ctttctaggt tcaaaagaag ttgtagtgtt 4816

atcaggcgtc ccataccttg tttttaatct cctgtttgtt gagtgcactg actgtgaaac 4876

ctttaccttt tttgttgttg ttggcaagct gcaggtttgt aatgcaaaag gctgattact 4936

gaaatttaag aaaaaggttc ttttttcaat aaatggttta ttttaggaaa gctc 4990

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<223> PCR Probe

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ccatgcgggg ctccgtgcac gg atg aga gaa gcc gct gcc gcg ctg gtc cct 172
Met Arg Glu Ala Ala Ala Ala Leu Val Pro
1 5 10

cct ccc gcc ttt gcc gtc acg cct gcc gcc gcc atg gag gag ccg ccg 220
Pro Pro Ala Phe Ala Val Thr Pro Ala Ala Ala Met Glu Glu Pro Pro

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                15                  20                  25

cca ccg ccg ccg ccg cca cca ccg cca ccg gaa ccc gag acc gag tca     268
Pro Pro Pro Pro Pro Pro Pro Pro Pro Glu Pro Glu Thr Glu Ser
            30                  35                  40

gaa ccc gag tgc tgc ttg gcg gcg agg caa gag ggc aca ttg gga gat     316
Glu Pro Glu Cys Cys Leu Ala Ala Arg Gln Glu Gly Thr Leu Gly Asp
        45                  50                  55

tca gct tgc aag agt cct gaa tct gat cta gaa gac ttc tcc gat gaa     364
Ser Ala Cys Lys Ser Pro Glu Ser Asp Leu Glu Asp Phe Ser Asp Glu
    60                  65                  70

aca aat aca gag aat ctt tat ggt acc tct ccc ccc agc aca cct cga     412
Thr Asn Thr Glu Asn Leu Tyr Gly Thr Ser Pro Pro Ser Thr Pro Arg
75                  80                  85                  90

cag atg aaa cgc atg tca acc aaa cat cag agg aat aat gtg ggg agg     460
Gln Met Lys Arg Met Ser Thr Lys His Gln Arg Asn Asn Val Gly Arg
                95                  100                 105

cca gcc agt cgg tct aat ttg aaa gaa aaa atg aat gca cca aat cag     508
Pro Ala Ser Arg Ser Asn Leu Lys Glu Lys Met Asn Ala Pro Asn Gln
            110                 115                 120

cct cca cat aaa gac act gga aaa aca gtg gag aat gtg gaa gaa tac     556
Pro Pro His Lys Asp Thr Gly Lys Thr Val Glu Asn Val Glu Glu Tyr
        125                 130                 135

agc tat aag cag gag aaa aag atc cga gca gct ctt aga aca aca gag     604
Ser Tyr Lys Gln Glu Lys Lys Ile Arg Ala Ala Leu Arg Thr Thr Glu
    140                 145                 150

cgt gat cat aaa aaa aat gta cag tgc tca ttc atg tta gac tca gtg     652
Arg Asp His Lys Lys Asn Val Gln Cys Ser Phe Met Leu Asp Ser Val
155                 160                 165                 170

ggt gga tct ttg cca aaa aaa tca att cca gat gtg gat ctc aat aag     700
Gly Gly Ser Leu Pro Lys Lys Ser Ile Pro Asp Val Asp Leu Asn Lys
                175                 180                 185

cct tac ctc agc ctt ggc tgt agc aat gct aag ctt cca gta tct gtg     748
Pro Tyr Leu Ser Leu Gly Cys Ser Asn Ala Lys Leu Pro Val Ser Val
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            190                 195                 200

ccc atg cct ata gcc aga cct gca cgc cag act tct agg act gac tgt     796
Pro Met Pro Ile Ala Arg Pro Ala Arg Gln Thr Ser Arg Thr Asp Cys
        205                 210                 215

cca gca gat cgt tta aag ttt ttt gaa act tta cga ctt ttg cta aag     844
Pro Ala Asp Arg Leu Lys Phe Phe Glu Thr Leu Arg Leu Leu Leu Lys
    220                 225                 230

ctt acc tca gtc tca aag aaa aaa gac agg gag caa aga gga caa gaa     892
Leu Thr Ser Val Ser Lys Lys Lys Asp Arg Glu Gln Arg Gly Gln Glu
235                 240                 245                 250

aat acg tct ggt ttc tgg ctt aac cga tct aac gaa ctg atc tgg tta     940
Asn Thr Ser Gly Phe Trp Leu Asn Arg Ser Asn Glu Leu Ile Trp Leu
                255                 260                 265

gag cta caa gcc tgg cat gca gga cgg aca att aac gac cag gac ttc     988
Glu Leu Gln Ala Trp His Ala Gly Arg Thr Ile Asn Asp Gln Asp Phe
            270                 275                 280

ttt tta tat aca gcc cgt caa gcc atc cca gat att att aat gaa atc    1036
Phe Leu Tyr Thr Ala Arg Gln Ala Ile Pro Asp Ile Ile Asn Glu Ile
        285                 290                 295

ctt act ttc aaa gtc gac tat ggg agc ttc gcc ttt gtt aga gat aga    1084
Leu Thr Phe Lys Val Asp Tyr Gly Ser Phe Ala Phe Val Arg Asp Arg
    300                 305                 310

gct ggt ttt aat ggt act tca gta gaa ggg cag tgc aaa gcc act cct    1132
Ala Gly Phe Asn Gly Thr Ser Val Glu Gly Gln Cys Lys Ala Thr Pro
315                 320                 325                 330

gga aca aag att gta ggt tac tca aca cat cat gag cat ctc caa cgc    1180
Gly Thr Lys Ile Val Gly Tyr Ser Thr His His Glu His Leu Gln Arg
                335                 340                 345

cag agg gtc tca ttt gag cag gta aaa cgg ata atg gag ctg cta gag    1228
Gln Arg Val Ser Phe Glu Gln Val Lys Arg Ile Met Glu Leu Leu Glu
            350                 355                 360

tac ata gaa gca ctt tat cca tca ttg cag gct ctt cag aag gac tat    1276
Tyr Ile Glu Ala Leu Tyr Pro Ser Leu Gln Ala Leu Gln Lys Asp Tyr
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        365                 370                 375

gaa aaa tat gct gca aaa gac ttc cag gac agg gtg cag gca ctc tgt    1324
Glu Lys Tyr Ala Ala Lys Asp Phe Gln Asp Arg Val Gln Ala Leu Cys
    380                 385                 390

ttg tgg tta aac atc aca aaa gac tta aat cag aaa tta agg att atg    1372
Leu Trp Leu Asn Ile Thr Lys Asp Leu Asn Gln Lys Leu Arg Ile Met
395                 400                 405                 410

ggc act gtt ttg ggc atc aag aat tta tca gac att ggc tgg cca gtg    1420
Gly Thr Val Leu Gly Ile Lys Asn Leu Ser Asp Ile Gly Trp Pro Val
                415                 420                 425

ttt gaa atc cct tcc cct cga cca tcc aaa ggt aat gag ccg gag tat    1468
Phe Glu Ile Pro Ser Pro Arg Pro Ser Lys Gly Asn Glu Pro Glu Tyr
            430                 435                 440

gag ggt gat gac aca gaa gga gaa tta aag gag ttg gaa agt agt acg    1516
Glu Gly Asp Asp Thr Glu Gly Glu Leu Lys Glu Leu Glu Ser Ser Thr
        445                 450                 455

gat gag agt gaa gaa gaa caa atc tct gat cct agg gta ccg gaa atc    1564
Asp Glu Ser Glu Glu Glu Gln Ile Ser Asp Pro Arg Val Pro Glu Ile
    460                 465                 470

aga cag ccc ata gat aac agc ttc gac atc cag tcg cgg gac tgc ata    1612
Arg Gln Pro Ile Asp Asn Ser Phe Asp Ile Gln Ser Arg Asp Cys Ile
475                 480                 485                 490

tcc aag aag ctt gag agg ctc gaa tct gag gat gat tct ctt ggc tgg    1660
Ser Lys Lys Leu Glu Arg Leu Glu Ser Glu Asp Asp Ser Leu Gly Trp
                495                 500                 505

gga gca cca gac tgg agc aca gaa gca ggc ttt agt aga cat tgt ctg    1708
Gly Ala Pro Asp Trp Ser Thr Glu Ala Gly Phe Ser Arg His Cys Leu
            510                 515                 520

act tct att tat aga cca ttt gta gac aaa gca ctg aag cag atg ggg    1756
Thr Ser Ile Tyr Arg Pro Phe Val Asp Lys Ala Leu Lys Gln Met Gly
        525                 530                 535

tta aga aag tta att tta aga ctt cac aag cta atg gat ggt tcc ttg    1804
Leu Arg Lys Leu Ile Leu Arg Leu His Lys Leu Met Asp Gly Ser Leu
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    540                 545                 550

caa agg gca cgt ata gca ttg gta aag aac gat cgt cca gtg gag ttt    1852
Gln Arg Ala Arg Ile Ala Leu Val Lys Asn Asp Arg Pro Val Glu Phe
555                 560                 565                 570

tct gaa ttt cca gat ccc atg tgg ggt tca gat tat gtg cag ttg tca    1900
Ser Glu Phe Pro Asp Pro Met Trp Gly Ser Asp Tyr Val Gln Leu Ser
                575                 580                 585

agg aca cca cct tca tct gag gag aaa tgc agt gct gtg tcg tgg gag    1948
Arg Thr Pro Pro Ser Ser Glu Glu Lys Cys Ser Ala Val Ser Trp Glu
            590                 595                 600

gag ctg aag gcc atg gat tta cct tca ttc gaa cct gcc ttc cta gtt    1996
Glu Leu Lys Ala Met Asp Leu Pro Ser Phe Glu Pro Ala Phe Leu Val
        605                 610                 615

ctc tgc cga gtc ctt ctg aat gtc ata cat gag tgt ctg aag tta aga    2044
Leu Cys Arg Val Leu Leu Asn Val Ile His Glu Cys Leu Lys Leu Arg
    620                 625                 630

ttg gag cag aga cct gct gga gaa cca tct ctc ttg agt att aag cag    2092
Leu Glu Gln Arg Pro Ala Gly Glu Pro Ser Leu Leu Ser Ile Lys Gln
635                 640                 645                 650

ctg gtg aga gag tgt aag gag gtc ctg aag ggc ggc ctg ctg atg aag    2140
Leu Val Arg Glu Cys Lys Glu Val Leu Lys Gly Gly Leu Leu Met Lys
                655                 660                 665

cag tac tac cag ttc atg ctg cag gag gtt ctg gag gac ttg gag aag    2188
Gln Tyr Tyr Gln Phe Met Leu Gln Glu Val Leu Glu Asp Leu Glu Lys
            670                 675                 680

ccc gac tgc aac att gac gct ttt gaa gag gat cta cat aaa atg ctt    2236
Pro Asp Cys Asn Ile Asp Ala Phe Glu Glu Asp Leu His Lys Met Leu
        685                 690                 695

atg gtg tat ttt gat tac atg aga agc tgg atc caa atg cta cag caa    2284
Met Val Tyr Phe Asp Tyr Met Arg Ser Trp Ile Gln Met Leu Gln Gln
    700                 705                 710

tta cct caa gca tcg cat agt tta aaa aat ctg tta gaa gaa gaa tgg    2332
Leu Pro Gln Ala Ser His Ser Leu Lys Asn Leu Leu Glu Glu Glu Trp
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715                 720                 725                 730

aat ttc acc aaa gaa ata act cat tac ata cgg gga gga gaa gca cag    2380
Asn Phe Thr Lys Glu Ile Thr His Tyr Ile Arg Gly Gly Glu Ala Gln
                735                 740                 745

gcc ggg aag ctt ttc tgt gac att gca gga atg ctg ctg aaa tct aca    2428
Ala Gly Lys Leu Phe Cys Asp Ile Ala Gly Met Leu Leu Lys Ser Thr
            750                 755                 760

gga agt ttt tta gaa ttt ggc tta cag gag agc tgt gct gaa ttt tgg    2476
Gly Ser Phe Leu Glu Phe Gly Leu Gln Glu Ser Cys Ala Glu Phe Trp
        765                 770                 775

act agt gcg gat gac agc agt gct tcc gac gaa atc atc agg tct gtt    2524
Thr Ser Ala Asp Asp Ser Ser Ala Ser Asp Glu Ile Ile Arg Ser Val
    780                 785                 790

ata gag atc agt cga gcc ctg aag gag ctc ttc cat gaa gcc aga gaa    2572
Ile Glu Ile Ser Arg Ala Leu Lys Glu Leu Phe His Glu Ala Arg Glu
795                 800                 805                 810

agg gct tcc aaa gca ctt gga ttt gct aaa atg ttg aga aag gac ctg    2620
Arg Ala Ser Lys Ala Leu Gly Phe Ala Lys Met Leu Arg Lys Asp Leu
                815                 820                 825

gaa ata gca gca gaa ttc agg ctt tca gcc cca gtt aga gac ctc ctg    2668
Glu Ile Ala Ala Glu Phe Arg Leu Ser Ala Pro Val Arg Asp Leu Leu
            830                 835                 840

gat gtt ctg aaa tca aaa cag tat gtc aag gtg caa att cct ggg tta    2716
Asp Val Leu Lys Ser Lys Gln Tyr Val Lys Val Gln Ile Pro Gly Leu
        845                 850                 855

gaa aac ttg caa atg ttt gtt cca gac act ctt gct gag gag aag agt    2764
Glu Asn Leu Gln Met Phe Val Pro Asp Thr Leu Ala Glu Glu Lys Ser
    860                 865                 870

att att ttg cag tta ctc aat gca gct gca gga aag gac tgt tca aaa    2812
Ile Ile Leu Gln Leu Leu Asn Ala Ala Ala Gly Lys Asp Cys Ser Lys
875                 880                 885                 890

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Asp Ser Asp Asp Val Leu Ile Asp Ala Tyr Leu Leu Leu Thr Lys His
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Gln Pro Val Lys Val Val Pro Gln Val Glu Thr Val Asp Thr Leu Arg
        925                 930                 935

agc atg cag gtg gat aat ctt tta cta gtt gtc atg cag tct gcg cat    3004
Ser Met Gln Val Asp Asn Leu Leu Leu Val Val Met Gln Ser Ala His
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ctc aca att cag aga aaa gct ttc cag cag tcc att gag gga ctt atg    3052
Leu Thr Ile Gln Arg Lys Ala Phe Gln Gln Ser Ile Glu Gly Leu Met
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act ctg tgc cag gag cag aca tcc agt cag ccg gtc atc gcc aaa gct    3100
Thr Leu Cys Gln Glu Gln Thr Ser Ser Gln Pro Val Ile Ala Lys Ala
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ttg cag cag ctg aag aat gat gca ttg gag cta tgc aac agg ata agc    3148
Leu Gln Gln Leu Lys Asn Asp Ala Leu Glu Leu Cys Asn Arg Ile Ser
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Asn Ala Ile Asp Arg Val Asp His Met Phe Thr Ser Glu Phe Asp Ala
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Glu Val Asp Glu Ser Glu Ser Val Thr Leu Gln Gln Tyr Tyr Arg Glu
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gca atg att cag ggg tac aat ttt gga ttt gag tat cat aaa gaa gtt    3292
Ala Met Ile Gln Gly Tyr Asn Phe Gly Phe Glu Tyr His Lys Glu Val
1035                1040                1045                1050

gtt cgt ttg atg tct ggg gag ttt aga cag aag ata gga gac aaa tat    3340
Val Arg Leu Met Ser Gly Glu Phe Arg Gln Lys Ile Gly Asp Lys Tyr
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Ile Ser Phe Ala Arg Lys Trp Met Asn Tyr Val Leu Thr Lys Cys Glu
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cta caa gca att gaa cct gcc ttt att tca gct tta cca gaa gat gac    3484
Leu Gln Ala Ile Glu Pro Ala Phe Ile Ser Ala Leu Pro Glu Asp Asp
    1100                1105                1110

ttc ttg agt tta caa gcc ttg atg aat gaa tgc att ggc cat gtc ata    3532
Phe Leu Ser Leu Gln Ala Leu Met Asn Glu Cys Ile Gly His Val Ile
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gga aaa cca cac agt cct gtt aca ggt ttg tac ctt gcc att cat cgg    3580
Gly Lys Pro His Ser Pro Val Thr Gly Leu Tyr Leu Ala Ile His Arg
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aac agc ccc cgt cct atg aag gta cct cga tgc cat agt gac cct cct    3628
Asn Ser Pro Arg Pro Met Lys Val Pro Arg Cys His Ser Asp Pro Pro
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Asn Pro His Leu Ile Ile Pro Thr Pro Glu Gly Phe Ser Thr Arg Ser
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atg cct tcc gac gcg cgg agc cat ggc agc cct gct gct gct gct gct    3724
Met Pro Ser Asp Ala Arg Ser His Gly Ser Pro Ala Ala Ala Ala Ala
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Ala Ala Ala Ala Val Ala Ala Ser Arg Pro Ser Pro Ser Gly Gly Asp
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Ser Val Leu Pro Lys Ser Ile Ser Ser Ala His Asp Thr Arg Gly Ser
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agc gtt cct gaa aat gat cga ttg gct tcc ata gct gct gaa ttg cag    3868
Ser Val Pro Glu Asn Asp Arg Leu Ala Ser Ile Ala Ala Glu Leu Gln
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Phe Arg Ser Leu Ser Arg His Ser Ser Pro Thr Glu Glu Arg Asp Glu
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gaa ctt cgg aca cta atc agc cag agt aaa gat act gct tct aaa cta    4012
Glu Leu Arg Thr Leu Ile Ser Gln Ser Lys Asp Thr Ala Ser Lys Leu
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gga ccc ata gaa gct atc cag aag tca gtc cga ttg ttt gaa gaa aag    4060
Gly Pro Ile Glu Ala Ile Gln Lys Ser Val Arg Leu Phe Glu Glu Lys
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agg tac cga gaa atg agg aga aag aat atc att ggt caa gtt tgt gat    4108
Arg Tyr Arg Glu Met Arg Arg Lys Asn Ile Ile Gly Gln Val Cys Asp
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acg cct aag tcc tat gat aat gtt atg cac gtt ggc ttg agg aag gtg    4156
Thr Pro Lys Ser Tyr Asp Asn Val Met His Val Gly Leu Arg Lys Val
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acc ttc aaa tgg caa aga gga aac aaa att gga gaa ggc cag tat ggg    4204
Thr Phe Lys Trp Gln Arg Gly Asn Lys Ile Gly Glu Gly Gln Tyr Gly
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Lys Val Tyr Thr Cys Ile Ser Val Asp Thr Gly Glu Leu Met Ala Met
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aaa gag att cga ttt caa cct aat gac cat aag act atc aag gaa act    4300
Lys Glu Ile Arg Phe Gln Pro Asn Asp His Lys Thr Ile Lys Glu Thr
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gca gac gaa ttg aaa ata ttc gaa ggc atc aaa cac ccc aat ctg gtt    4348
Ala Asp Glu Leu Lys Ile Phe Glu Gly Ile Lys His Pro Asn Leu Val
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Arg Tyr Phe Gly Val Glu Leu His Arg Glu Glu Met Tyr Ile Phe Met
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Asn Val Leu His Glu His Gly Ile Val His Arg Asp Ile Lys Gly Ala
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aat atc ttc ctt acc tca tct gga tta atc aaa ctg gga gat ttt gga    4588
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Cys Ser Val Lys Leu Lys Asn Asn Ala Gln Thr Met Pro Gly Glu Val
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aac agc acc ctg ggg aca gca gca tac atg gca cct gaa gtc atc act    4684
Asn Ser Thr Leu Gly Thr Ala Ala Tyr Met Ala Pro Glu Val Ile Thr
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cgt gcc aaa gga gag ggc cat ggg cgt gcg gcc gac atc tgg agt ctg    4732
Arg Ala Lys Gly Glu Gly His Gly Arg Ala Ala Asp Ile Trp Ser Leu
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Gly Cys Val Val Ile Glu Met Val Thr Gly Lys Arg Pro Trp His Glu
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Tyr Glu His Asn Phe Gln Ile Met Tyr Lys Val Gly Met Gly His Lys
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